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Protocol for Large-Scale Collection, Processing, and Storage of Seeds of Two Mesohaline Submerged Aquatic Plant Species

by Steve Ailstock and Deborah Shafer

PROBLEM: Seeds offer an efficient and cost-effective method for providing new plants for large-scale plantings and hence are used for the production of all major domesticated crop plants. Similarly, seed propagation offers the most cost-effective approach for restoring large, genetically diverse, self-maintaining populations of underwater grasses. However, their use in submerged aquatic vegetation restoration has been limited to date. Recently, the harvest and sowing of seeds of eelgrass (*Zostera marina*) has emerged as a viable means of planting and restoring large acreages (Pickerell et al. 2005, Orth et al. 2003, Granger et al. 2002). While protocols for the large-scale collection, processing, storage, and subsequent distribution of seeds of this species are relatively well-developed, similar protocols are lacking for other submerged aquatic plant species.

PURPOSE: A previous pilot study (Ailstock and Shafer 2004) established the seed reproductive potential of two species of submersed aquatic angiosperms, *Ruppia maritima* (widgeon grass) (Figure 1) and *Potamogeton perfoliatus* (redhead grass), that predominate in the mesohaline reaches of the mid-Chesapeake Bay. This paper outlines a system for the collection, processing, storage, and germination of seeds in quantities sufficient to restore underwater grass habitats on a scale of hectares. Although this system was developed for *P. perfoliatus* and *R. maritima*, the same procedures could also be used to develop protocols for seeds of other aquatic plant species for use in large-scale restoration projects.

Figure 1. A one-gram plant sample of *R. maritima* seeds

BACKGROUND: The evolution of seeds conferred a huge selective advantage to the spermatophytes and became the dominant reproductive unit for dispersing plants to new habitats. Embryos formed in seeds are better protected and have more abundant food reserves than those in other plant groups like the bryophytes and pteridophytes (Batygina 2002, Fernald 1970). Auxiliary seed tissues provide mechanisms for efficient embryo (seed) dispersal and the regulation of the timing of initial seedling growth. The evolution of flowering plants extended the potential for regulating growth and improved the efficiency of gamete transfer via pollination (Willis and

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McElwain 2002, Raghavan 2000). The relatively small size of seeds allows plants to produce large numbers of potential offspring that vastly exceed those that could be formed by other reproductive pathways. For example, the small 1-g sample of *Ruppia maritima* in Figure 1 has 38 seeds. Taken collectively, these adaptations improve the reproductive success of plants by imparting the ability to minimize competition between generations of plants through the combination of improved seed dispersal, enhanced longevity of viable seeds over periods of years, and by regulating the timing of seed germination to periods favorable to the establishment of a new generation of reproductive plants (Hemsley and Poole 2004, Copeland and McDonald 2001, Simpson et al. 1989).

The adaptations which proved so advantageous for the success of terrestrial angiosperms, coupled with the submersed growth habit of underwater grasses, pose challenges for the development of propagation protocols for plants used for environmental restoration projects. For example, most plants produce mature seeds sequentially over time so that at least some seeds detach under conditions that favor their effective dispersal and subsequent establishment. In contrast, propagation systems like those employed by agriculture are more efficient if seeds mature uniformly and can be harvested in quantity at a particular time (Benech-Arnold and Sanchez 2004, Copeland and McDonald 2001). The approach used in agriculture to selectively breed for uniformity of plant growth and development is contrary to the goals of ecological restoration where the objective is the successful establishment of organisms that possess all of the genetic diversity inherent in the parental stock, including such properties as asynchronous maturation. Moreover, the physical constraints of working with underwater flowering plants pose unique challenges not found in the working environment of terrestrial or emergent aquatic plants (e.g. development of efficient seed harvest techniques). Still, there are shared requirements for all propagation strategies employing seeds. These include seed harvest, storage, viability testing, germination, and seedling growth.

SEED HARVEST PROCEDURES: Once vigorous, healthy SAV populations are located, they must be monitored for floral development and seed formation since both the abundance of seeds and the timing of seed development can fluctuate significantly between years according to ambient environmental conditions. Stands of *R. maritima* that produce copious seeds one year may produce few seeds in subsequent years despite apparently abundant vegetative growth. Similarly, *P. perfoliatus* may exhibit differences in seed formation, sometimes with few seeds produced in different years and, in others, abundant seeds produced in two distinct flowering cycles, one early in the summer, the other toward the middle to end of the seasonal growth cycle (Ailstock and Shafer 2004, Spencer and Ksander 1996, Titus and Hoover 1991). Optimal seed formation for several estuarine SAV species in the mid-Chesapeake Bay occurs in July and August. This period coincides with the formation of tropical depressions that can significantly elevate site energetics. Since the stems naturally may tend to weaken and detach during and immediately after peak seed formation, the appearance of storms can eliminate an otherwise promising collection site literally overnight. Thus, multiple collection sites, some in protected coves, should be identified in advance of seed harvesting. Collecting throughout the reproductive cycle optimizes diversity and ensures that phenotypes that mature early and late are captured in the collection process. While this will reduce the efficiency of seed harvest, those seeds that are collected will include a range of phenotypes, which is important for improving establishment under the variable conditions of the ambient environment.

Figure 2. Seed-bearing wrack on shoreline

There are two options for seed collection. The first utilizes seeds retained in detached stems that accumulate as wrack along shorelines following anthesis (Ailstock and Shafer 2004). This material offers the advantage of a seed source that would otherwise be unavailable for natural reproduction (Figure 2). One disadvantage of wrack is that most seeds have been dispersed; therefore, per-unit seed yields are lower than that of material harvested during active growth. A second disadvantage is that the availability of wrack is heavily influenced by weather. Sites along the west-facing shorelines of protected coves can yield abundant wrack in a year where prevailing west winds are light. The same location may yield little if any wrack when storms disperse the broken stems to open-water environments.

A more reliable method for harvesting seeds is to collect seeds directly from actively growing beds prior to stem detachment. For *R. maritima* and *P. perfoliatus*, approximately 75 to 85 percent of the floral structures are produced in the upper third of the stems regardless of water depth (Ailstock, unpublished data). Therefore, the topmost one-third of the seed-bearing photosynthetic stems can be hand-harvested and placed in porous aquaculture baskets (Figures 3 and 4). This technique leaves the majority of the photosynthetic stems in place and appears to have negligible effect on the subsoil roots and rhizomes. Since hand harvesting is used, many reproductive stems remain intact, thereby minimizing potential adverse effects on the population by leaving considerable amounts of seed in the collection area. Finally, to help minimize selection for uniform seed maturation, the same populations are used for three or more collections during the seasonal reproductive cycle of the plants (Ailstock and Shafer 2004).

Figure 3. Harvesting seed-bearing stems

Figure 4. Basket of seed-bearing stems

Figure 5. Transporting filled baskets

Although collection efficiency will vary with stem densities of the selected populations, most sites allow for the collection of a packed 10-gal basket in under 15 minutes with modest effort. Once the baskets are dewatered by gravity, the yield is approximately 20 lb of seed-bearing stems for both *R. maritima* and *P. perfoliatus*. The baskets are then transported to the processing facility (Figure 5) and the collected material placed in large plastic aquaculture tanks. The stem material should be spread in the tanks to a depth of about 1 ft and monitored for temperature as a safeguard against excessive temperatures caused by composting of the heavily hydrated organic mixture that can effectively pasteurize and kill the seeds prior to further processing.

SEED PROCESSING: Reproductive structures in the collected stem material occur in various stages of development ranging from immature flowers to seed stalks from which mature seeds have already detached. Seed processing is a method of isolating the mature seeds from the stems and other less-developed reproductive structures (Benech-Arnold and Sanchez 2004, Raghavan 2000). An old-fashioned wringer washing machine (Speed Queen, Lehman Hardware & Appliances, Inc., Kidron, Ohio) provides the agitation needed to detach and collect seeds in quantity from the collected material. A mesh grid made from hardware cloth sized to allow seeds to pass through (0.5- to 0.25-in. mesh) is placed in the bottom of the machine above the gravity feed drain. Stem material is placed in the tub in tap water and agitated for 1 to 5 minutes, depending on the results of trial runs assessing the time of agitation and yield of full size seed. After agitation, the tub material is dewatered by passage through the washer's wringer and placed back in the aquaculture tubs to allow for subsequent ripening of seeds during an additional 4 to 5 days of cool storage. After several batches of bulk material are agitated and removed, the tub is drained. Seeds and seed size debris are collected in mesh bags placed over the drain hose. The general process is shown in Figure 6. The isolation process is repeated using the stored bulk material initially processed to collect additional seeds that mature during temporary storage. Depending on the desired use of the seeds, they may be further processed through a series of washings and screenings to remove residual vegetative debris.


Figure 6. Seed processing

SEED STORAGE AND GERMINATION: Several objectives should be met when storing seeds of underwater grasses that are intended for use in restoration projects (Young and Young 1986). First, storage conditions must provide an environment that allows seeds to remain viable and dormant, since embryo death, or premature germination, will negate their use for restoration.

Second, it is often desirable to provide conditions that meet the plant's requirements for subsequent germination. For example, seeds of many native plants must be stratified, scarified, or leached to overcome the dormancy mechanisms plants exploit to adjust the timing of germination to favorable conditions for plant establishment. Third, determining the particular adaptations that plants use to regulate germination allows propagators the option of storing some seeds so that some are capable of immediate germination, while germination for others will be delayed and occur over long periods. Such a mixture of treatments provides an important hedge against weather-related fluctuations that often occur after seeds have been planted.

Three methods are currently used to store and disperse seeds for restoration projects involving all species. Two require either no storage or temporary storage under the ambient conditions to which wild populations are generally exposed. The first method deploys the freshly collected floral stems in mesh bags attached to floats that allow seed to be dispersed under the ambient conditions immediately after collection (Pickerell et al. 2005). The second involves storage and incubation of the seeds in flow-through aquaculture tanks to facilitate ripening and distribution at times known to be conducive to seed germination and seedling establishment (Tom Parham, personal communication). Storage is of relatively short duration and is generally limited to ambient or cold treatments. A third method focuses on long-term storage. This method affords the opportunity to have seeds available whenever they are needed.

R. maritima and *P. perfoliatus* occur in environments that are known to fluctuate widely in temperature, salinity, and oxygen. In order to determine which combinations of conditions are most conducive to seed germination and growth, seeds of *P. perfoliatus* harvested in July and August were processed and stored under various combinations of temperature (4° and 21 °C), salinity (0, 10, and 15 ppt), and oxygen (aeration, no aeration) for 9 months. During the first 6 months of storage, random samples of 200 seeds each were extracted monthly to assess germination rates under storage conditions. After 6 and 9 months of storage, germination was assessed weekly for 3 weeks using samples of 50 seeds each placed in ambient storage salinities (0, 10, and 15 ppt) at a temperature of 21 °C and light levels of 77 $\mu E/m^{-2}/s^{-1}$.

The results of these experiments indicate that *P. perfoliatus* seeds must be stored under cold conditions to remain viable in storage and capable of germination when transferred to inductive temperatures. Longer cold storage improved germination without affecting viability. Aeration during storage was also important for retaining the viability of stored seeds. Germination of seeds stored at 4 °C with aeration was rapid with most seeds germinating within 7 days of inductive treatments. Salinity is also an important factor for regulating the time of germination to coincide with low salinities outside the normal range of salinity preferred by adult plants. Only 7.5 percent of the seeds stored at 15 ppt salinity germinated within 3 weeks if transferred to the same salinity at 21 °C. In contrast, those same seeds show 85.5 percent germination when transferred to 0 salinity at 21 °C. Such salinities generally occur following severe storms with heavy precipitation, conditions known to stress or cause mortality of established plants (Barko et al. 1991). Thus, *P. perfoliatus* uses salinity cues to promote seed germination at times when mature plants are stressed, a mechanism that minimizes competition between generations of plants by providing an abundance of replacement plants at times when suitable habitats are most abundant. Thus, under normal salinity conditions, a small proportion of the seeds may exhibit slow, continuous germination, while most will remain dormant until an event that lowers salinity and favors seedling establishment.

Table 1
Optimum Conditions for Storage and Germination of *P. perfoliatus* Seeds

Storage Temperature	Aeration During Storage	Storage Period	Germination Salinity
Cold storage (4 °C)	Yes	9 months	0 ppt

Seeds of *R. maritima* were also harvested and processed in July and August 2004 and stored under conditions similar to those described for *P. perfoliatus*. *R. maritima*, which enjoys a much wider distribution than *P. perfoliatus*, exhibited greater phenotypic plasticity. Like *P. perfoliatus*, germination under inductive conditions was rapid with most germination occurring within 1 week. Cold storage improved germination at all salinities tested, but also induced premature germination during storage. This premature germination reduces effective seed yield for restoration since these seeds are lost for plantings. Future efforts are focusing on identification of a more precise set of storage conditions that will minimize premature germination without significant loss in germination potential. Although *R. maritima* is capable of germinating under a wide array of salinities, the best germination was achieved with storage at higher salinities that are consistent with the preferred salinities of mature plants (Green and Short 2003, Koch and Dawes 1991). However, the highest germination rate was achieved when seeds stored at 21 °C at 15 ppt were placed in fresh water. This suggests that *R. maritima,* like *P. perfoliatus*, possesses an adaptation to encourage rapid seed germination after heavy precipitation that reduces salinities below that preferred by mature plants. Thus, *R. maritima* also exploits salinity cues as a mechanism to reduce competition among generations timed to coincide with conditions that stress mature plants. Although 20 to 40 percent of seeds stored at 4 °C may germinate during storage, the germination upon removal from storage can reach 70 percent when seeds are stored at a salinity of 15mg/l. Ecotypic variation with respect to this adaptation is unknown and should be investigated.

POTENTIAL FOR FURTHER USE: Seeds are the only propagules that contribute to genetic diversity between generations of plants. Care must be taken to sacrifice some propagation efficiency to preserve maximum genetic diversity of parental stock. It is in this area that seed technology for producing native plants differs most from that used in the production of domesticated plants, where uniformity in all aspects of production and in plant phenotype is desired as a way of reducing production costs to a minimum. With the possible exception of such plants as *Zostera marina* and *Thalasia testudinum*, information on the variation in storage and germination requirements of the seeds of most underwater grasses is sparse (Orth et al. 1994, 2000; Moore et al. 1993; Durako and Moffler 1984; Orth and Moore 1983). However, applying these protocols to other species and ecotypes can yield important information on the diversity of these requirements and help shape production methods that will preserve this important aspect of underwater grass diversity.

These data also point out the importance of experimental studies to determine the optimum conditions for seed storage and germination. The application of this information to restoration is significant. In many cases, optimum conditions for seed storage and germination are assumed to be the same as those of the adult plants. This study shows that some aquatic plants have evolved strategies to minimize competition between generations and ensure vigorous recovery of plant stocks if adult populations become stressed due to unfavorable environmental conditions.

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